Journal of Biosciences and Medicines, 2022, 10, 1-13
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Methicillin-Resistant *Staphylococcus aureus* May Also Be Resistant to Clindamycin and Vancomycin

Miriam G. U. Anejionu[1,2], Angus N. Oli[1], Chijioke E. Ezeudu[3*], Ogochukwu I. Ezejiofor[4], Joseph Ezeogu[5], Anthony A. Attama[6], Vincent C. Okore[2]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria
[2]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria
[3]Department of Paediatrics, Nnamdi Azikiwe University, Awka, Nigeria
[4]Dermatology Unit, Department of Medicine, Nnamdi Azikiwe University, Awka, Nigeria
[5]Department of Paediatrics, Federal Medical Centre, Owerri, Nigeria
[6]Drug Delivery Research Unit, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria
Email: gu.anejionu@unizik.edu.ng, an.oli@unizik.edu.ng, *ce.ezeudu@unizik.edu.ng, oi.ezejiofor@unizik.edu.ng, jezeogu@yahoo.com, anthony.attama@unn.edu.ng, vincent.okore@unn.edu.ng

How to cite this paper: Anejionu, M.G.U., Oli, A.N., Ezeudu, C.E., Ezejiofor, O.I., Ezeogu, J., Attama, A.A. and Okore, V.C. (2022) Methicillin-Resistant Staphylococcus aureus May Also Be Resistant to Clindamycin and Vancomycin. *Journal of Biosciences and Medicines*, **10**, 1-13.
https://doi.org/10.4236/jbm.2022.108001

Received: June 25, 2022
Accepted: July 30, 2022
Published: August 2, 2022

Abstract

Objectives: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a global superbug widely distributed in hospitals, communities and livestock settings. This study investigated the presence and molecular characterization of MRSA co-resistance to clindamycin and vancomycin in the southeastern region of Nigeria. The susceptibility of these organisms to other selected antibiotics was also investigated. **Method:** Biological samples were obtained from consenting patients from three establishments in Enugu, Nigeria and cultured for isolation and purification. The pure isolates were subjected to antimicrobial susceptibility profiling using conventional antibiotics. The genomic DNAs of the pure isolates were isolated using the Promega genomic DNA purification kit while the antibiotic resistance genes (*mecA*) genes were identified using a multiplex polymerase chain reaction. Also, the minimum inhibitory concentration of the clindamycin and vancomycin antibiotics was determined as well as their combined activity on the MRSA isolates. **Results:** A large proportion (71%) of the MRSA isolates was from urine samples and then from the High Vaginal Swab (19%). All the isolates were resistant to cloxacillin while 95% were resistant to ciprofloxacin. MRSA isolates demonstrated resistance to clindamycin (with MIC of 23.44 - 250 μg/ml) and to vancomycin (with MIC

of 62.5 - 250 μg/ml). The isolated MRSA also demonstrated multidrug-resistant traits. The combined effects of vancomycin and clindamycin against different species of MRSA exhibited additive, antagonistic and indifferent effects and none had a synergistic effect. Multiplex Polymerase Chain Reaction revealed that the majority of the strains were positive for the 162-bp internal fragment of the *mecA* gene of MRSA and basically displayed SCC*mec* type III, indicating that they were multidrug-resistant and hospital-acquired. **Conclusion:** Clindamycin and vancomycin-resistant MRSA infections are also within the Eastern region of Nigeria as found in other countries of the world. This superbug, therefore, may require drastic and urgent measures to curtail its spread and attendant healthcare challenges like outbreaks of infections. In addition, strict adherence to antibiotic policy and continuous surveillance is highly advocated.

Keywords

Methicillin-Resistant, *Staphylococcus aureus*, Vancomycin, Clindamycin

1. Introduction

The Methicillin-resistant *Staphylococcus aureus* (MRSA) superbug is a strain of *Staphylococcus aureus* bacteria that has passed through some mutations which confers it some ability to resist most classes of antibiotics. It does so by producing an enzyme that cleaves part of the chemical structure of those antibiotics thereby causing severe morbidity, mortality, prolonged hospital stay and financial burden [1] [2] [3] due to treatment failures.

MRSA infections can be Healthcare-associated [4] [5], Community-associated [6] [7] [8] [9] [10] or Livestock-associated [11] [12]. MRSA has been defined according to the symptoms in patients they infect as well as by their treatment, antibiotic susceptibility and genetic characteristics [1] [4].

For Livestock-associated MRSA, the most likely transmission route is by direct contact with infected livestock or their environment [13] [14]. Through these sources, MRSA can move into hospitals, causing severe infections and outbreaks [15]. MRSA infection can cause skin and soft tissue infections, bacteremia, osteomyelitis, urinary tract infection, endocarditis, and pneumonia [1] [2].

MRSA infections are commonly treated with vancomycin, teicoplanin, cephalexin, clindamycin, trimethoprim-sulfamethoxazole, doxycycline, fusidic acid and linezolid depending on the strain's susceptibility profile [16] [17]. However, some studies outside Nigeria have documented co-clindamycin-vancomycin-resistant MRSA in certain parts of the world [18] [19] [20] [21]. Currently, there is no study on MRSA resistance to clindamycin and vancomycin in Southeastern Nigeria. Also, some researchers investigated the acquisition of antibiotic resistance genes by methicillin-resistant strains and traced it to antibiotic consumption in hospitals [20] [21] [22]. As such, characterization of resistance genes may aid in

tracing infection sources and the spread of resistance traits. Hence, this study investigated the presence and molecular characterization of MRSA isolated in Enugu, the southeast region of Nigeria and their level of susceptibility to other antibiotics was also revealed.

Primarily, this study is aimed to empirically determine the level of MRSA strains co-resistant to vancomycin and clindamycin in Enugu, Nigeria and molecularly characterize them.

2. Materials and Methods

2.1. Study Design

This was a cross-sectional study conducted at two tertiary hospitals and molecular pathology laboratory all in Enugu, Southeast, Nigeria.

2.2. Study Area

The University of Nigeria Teaching Hospital (UNTH), Annunciation Specialist Hospital and Safety Molecular Pathology Laboratory Services Ltd. are all located in Enugu, Southeast, Nigeria. Enugu is the largest city and the capital of Enugu State, Nigeria, with an estimated population of 795,000 as of 2021.

2.3. Sample Collection and Processing

A total of one hundred *S. aureus* samples previously isolated from clinical samples at University of Nigeria Teaching Hospital, Annunciation Specialist Hospital and Safety Molecular Pathology Laboratory Services Ltd, all located in Enugu, Southeast of Nigeria were used for the study. The clinical samples were: urine, seminal fluid, vaginal discharge, urethral smears, skin scrapings, catheter tips, sputum and, then swab samples from endocervix, ears, throat, wounds and eyes.

The agar media used include: "Mueller Hinton Agar (MHA), Mannitol salt agar (MSA)" and "Methicillin-Resistant *Staphylococcus aureus* (MRSA) Agar Base (Acumedia, Michigan, USA)" while the broth media used were: Lactose broth, Glucose broth, Brain Heart Infusion (BHI) and Sucrose broth. Manufacturer's instructions were followed strictly during each of the media preparation. Vancomycin and clindamycin (Hospira, Inc. Lake Forest, USA) and then cloxacillin sodium (Nichben Pharm. Co. Nig Ltd.) were obtained and used as pure drugs. Antimicrobial sensitivity discs containing cefixime (5 µg), co-trimoxazole (30 µg), clindamycin (10 µg), gentamycin (10 µg), ciprofloxacin (5 µg), cloxacillin (30 µg), ofloxacin (5 µg), ceftriaxone (30 µg), norfloxacin (10 µg), and erythromycin (10 µg) (Oxoid, UK) were also used to determine the susceptibility profile. Phenol red and glycerol, Ethidium bromide solution (Sigma-Aldrich), TAE buffer, blue/orange loading dye (Promega Madison, USA), agarose gel, 100 bp DNA ladder (Promega Madison, USA) were the reagents used. Promega genomic DNA purification kits were also used.

The isolates were inoculated onto MRSA agar plates, each containing cloxacillin sodium 600 µg/ml in 1 litre agar and incubated for 24 h at 37˚C [23]. The co-

lonial growths were stored in aliquots containing 85% glycerol mixed with 15% distilled water at 4˚C. Before use, an aliquot of the test isolates were cultured again onto fresh 1 litre of MRSA agar that contained 600 µg/ml of cloxacillin sodium and then incubated for 24 h at 37˚C for reactivation. Reactivated cultures were standardized by growing the organism in oxygen-rich shaker water bath at 37˚C for 16 ± 2 h to a cell density of 2.0×10^8 cfu/ml [24].

The MIC of clindamycin, vancomycin, gentamycin, ciprofloxacin and ceftriaxone was evaluated using macro-broth dilution method. Briefly, two-fold serial dilutions of standardized drugs in BHI were made and mixed with 0.1 ml standardized MRSA cultures in tubes. After 20 mins, the tubes were incubated at 37˚C for 24 h. The microbial growths were examined visually. The MIC was recorded (after duplicate readings) as the lowest concentration of the drugs that inhibits the growth of the MRSA after the 24 h incubation period [23] [24].

The antibiogram study of the MRSA isolates with other conventional antibiotics was determined using the Kirby-Bauer disc diffusion technique (described as follows). 0.1 ml of standardized MRSA cultures were diluted with sterile saline to get the turbidity match of 0.5 McFarland standards and dispensed unto dried agar plates. These were dispersed evenly onto the agar surface using sterile swab stick to make a bacterial lawn. Inoculated plates were allowed to dry for 15 mins and the various antibiotic discs were aseptically placed on the inoculated plates at 15 mm away from the edge of the plates. The plates were allowed a further drying period of 30 mins and then incubated for 24 h at 37˚C. After incubation, zone of clearance/inhibition was observed and the diameter of the zone was measured, recorded and compared with a standard for each drug. The isolates were recorded as resistant, intermediate or susceptible based on the standard interpretative chart as described by the Clinical Laboratory Standard Institute (CLSI) [25].

Different solutions of clindamycin phosphate and vancomycin hydrochloride were prepared with water for injection. Each solution contained double the MIC of each drug. The two drugs solutions were mixed in continuous variation proportions of checkerboard model [26]. Each mixed proportion was then diluted (in triplicates) with normal saline in two-fold serial dilution up to 7 dilutions in aseptic test-tubes. Then, 0.1 ml of 0.5 McFarland standard MRSA cultures were inoculated into each tube and incubated at 37˚C for 24 h. The Fractional Inhibitory Concentration (FIC) of all the combination ratios of the drugs was determined while the FIC value for each drug was computed using the formula [26]:

$$\mathrm{FIC_A} = \frac{\text{MIC of drug A in combination with drug B}}{\text{MIC of drug A alone}}$$

$$\mathrm{FIC_A} = \frac{\text{MIC of drug B in combination with drug A}}{\text{MIC of drug B alone}}$$

$$\text{FIC Index} = \mathrm{FIC_A} + \mathrm{FIC_B}$$

The FIC index is interpreted as: “FIC index < 1.0 means Synergism, =1 means additivity, >1, but less than 2 means indifference while ≥2 means antagonism”

[26].

The chromosomal DNA of all the MRSA isolates was extracted and quantified using the Promega chromosomal DNA purification kit and as laid-out in the manufacturer's instruction booklet (Promega Madison, USA). The presence of the antibiotic resistance genes (the 310 base pair (bp) PCR product of *mecA* gene) genes were investigated using multiplex polymerase chain reaction (PCR), the primers being: "forward (5'-TGG CTA TCG TGT CAC AAT CG-3') and reverse (5'-CTG GAA CTT GTT GAG CAG AG-3')". The multiplex PCR amplifications were undertaken in 30 µl reaction volume (reaction mixture) comprising of 12.5 µl of master mix, 7.5 µl of primer mix and 8 µl of gDNA in 2 µl dyes. Also, 100 bp DNA ladder and *mecA* positive strain were included to serve as the positive control. The PCR cycling conditions (Thermal profile) were in the order: Initial denaturation at 94˚C lasted 10 minutes; 35 cycles of amplification at 94˚C lasted for 30 seconds; annealing at 53˚C lasted for 30 seconds; extension at 72˚C lasted for a minute and finally, extension at 72˚C lasted for 5 minutes. Amplification products were analyzed on a 2.0% agarose gel electrophoresis prepared by 2 g of gel to 100 mls of TAE buffer for efficient separation [27].

3. Results

Our result shows that MRSA was obtained in 80% of *S. aureus* isolates. The highest numbers (70.65%) of the MRSA isolates were obtained from urine samples followed by high vaginal swab (19.06%). The rest were; urethral swab (3.34), semen (2.14), wound swab (2.09), sputum (1.31), ear swab (0.31), endocervical swab (0.31), throat swab (0.21), catheter tip (0.21), eyes-swab (0.21) and skin scraping (0.10).

The gram-stained pictures in **Figure 1** show that all the isolates were gram- positive cocci that occurred in singles, pairs and as grape-like clusters lacking definite shapes. The biochemical tests showed that all the isolates coagulated blood plasma, bubbles upon mixing with H_2O_2 and fermented glucose, sucrose, lactose and mannitol.

The inhibitory effects of these standard drugs (vancomycin and clindamycin) against isolates of MRSA reveal that both drugs have activity against MRSA but at a higher concentration, indicating resistance of the organisms to the drugs.

Table 1 shows the resistance of organisms to various drugs, whereas 100% of the isolates were resistant to cloxacillin 95% were resistant to ciprofloxacin. It was also observed that at least 70% of the isolates were resistant to every other antibiotic while, 40% were sensitive to more than one class of antibiotics (**Table 2**). In addition, 15% of isolated MRSA strains were resistant to all the antibiotics (MDR) and none was fully susceptible to all the tested antibiotics (**Table 2**). Invariably, all the MRSA could be taken as multidrug-resistant (MDR) from these results.

The MIC result revealed that these drugs are above the susceptibility range, indicating the resistance character of the MRSA isolates (**Table 3**). The isolates were, however, susceptible to ceftriaxone.

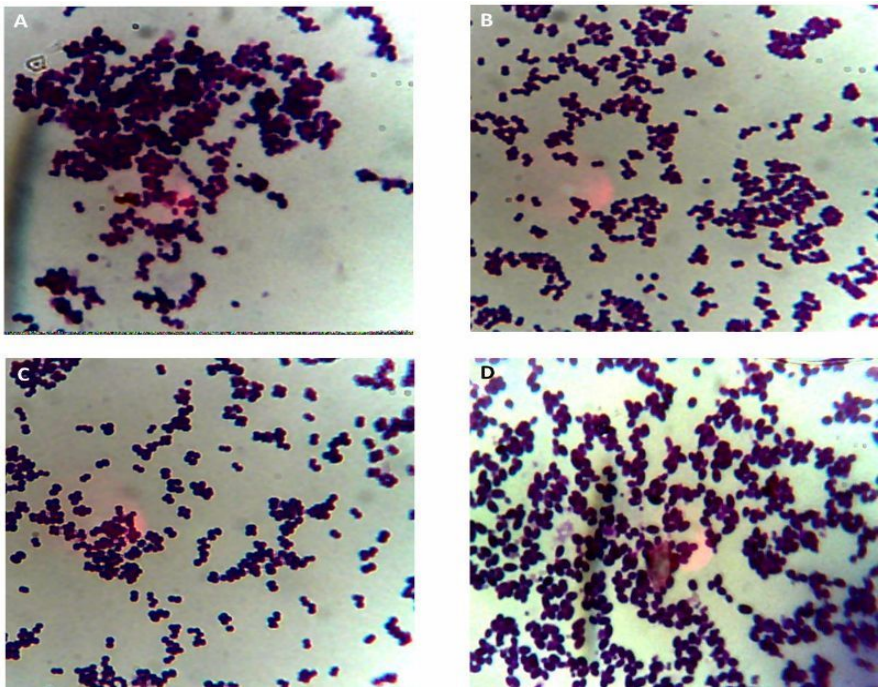

Figure 1. Gram stained pictures of MRSA isolated from different clinical samples ((A) urine, (B) high vaginal swab (HVS), (C) wound swab, (D) semen).

Table 1. The antibiogram of MRSA to various antibiotics.

Antimicrobial	Sensitive	% Sensitive	Resistance	% Resistance
Gentamicin 10 µg/ml	12	30	28	70
Ofloxacin 5 µg/ml	8	20	32	80
Clindamycin 10 µg/ml	10	25	30	75
Ceftriaxone 30 µg/ml	8	20	32	80
Cloxacillin 30 µg/ml	0	0	40	100
Cefixime 5 µg/ml	4	10	36	90
Norfloxacin 10 µg/ml	6	15	34	85
Ciprofloxacin 5 µg/ml	2	5	38	95
Erythromycin 10 µg/ml	8	20	32	80
Septrin 30 µg/ml	10	25	30	75

The combinations of vancomycin and clindamycin against different species of MRSA exhibited additive, antagonistic and indifferent effect. None had synergistic effect (Figure 2).

Multiplex PCR for MRSA typing is shown in Figure 3. In our work, majority of the strains harbour the 162 bp internal fragment of the *mecA* gene and so confirms that they are MRSA. This result reveals that MRSA isolates basically displayed SCC*mec* type III with four bands of 303, 414, 303 and 414 bp.

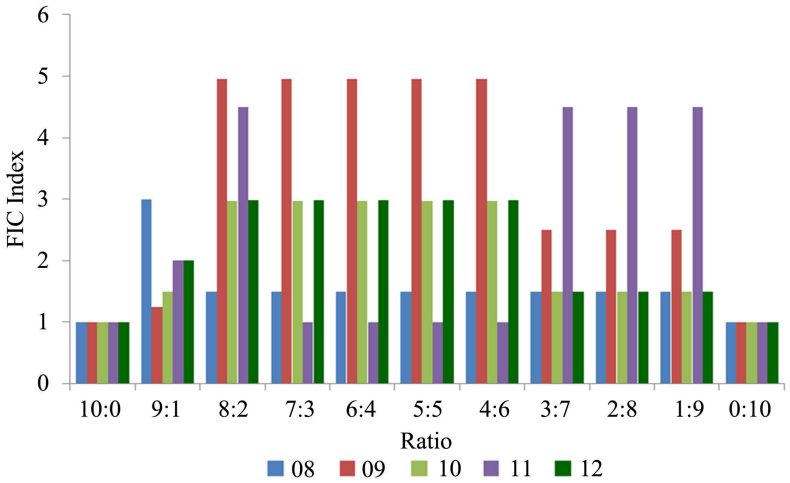

Figure 2. Combination of vancomycin and clindamycin against isolates of MRSA.

Table 2. Antibiotic sensitivity and resistance pattern of MRSA to various antibiotics.

Isolates	No of sensitive drugs	No of resistant drugs	Inference	Isolates	No of sensitive drugs	No of resistant drugs	Inference
1	1	9	GN only was Sensitive	21	6	4	GN, CE, NB, E, CT, CXM were sensitive
2	1	9	CT only was Sensitive	22	4	6	GN, E, CT CXM were Sensitive
3	1	9	Only CD was Sensitive	23	3	7	GN, CT, CXM were sensitive
4	1	9	Only E was Sensitive	24	4	6	NB, OF, CT, CXM were Sensitive
5	4	6	GN, CD, CE, NB were sensitive	25	2	8	CD, OF, were Sensitive
6	1	9	OF only was Sensitive	26	0	10	All were Resistant
7	3	7	E, OF, CXM are Sensitive	27	2	8	CD, CE were Sensitive
8	2	8	GN, CD were Sensitive	28	1	9	GN was Sensitive
9	6	4	GN, CD, CE, NB, OF, CT were Sensitive	29	1	9	CD was Sensitive
10	2	8	E, CXM were Sensitive	30	1	9	CD was Sensitive
11	1	9	CXM only was Sensitive	31	1	9	NB was Sensitive
12	1	9	CXM only was Sensitive	32	1	9	CXM was Sensitive
13	0	10	All were resistant	33	1	9	CD was sensitive
14	0	10	All were resistant	34	1	9	CIP was Sensitive
15	2	8	CT, CXM were Sensitive	35	0	10	All were Resistant
16	4	6	GN, CD CIP, CT were sensitive	36	1	9	OF only was Sensitive
17	0	10	All were resistant	37	0	10	All were Resistant
18	1	9	NB was sensitive	38	1	9	E was sensitive
19	1	9	GN was sensitive	39	2	8	GN, E were Sensitive
20	2	8	OF, E are sensitive	40	2	8	GN, OF were Sensitive

Drugs Key: AP = Cloxacillin (30 µg), GN = Gentamycin (10 µg), CD = Clindamycin (10 µg), CIP = ciprofloxacin (5 µg), CE = Cefixime (5 µg), NB = Norfloxacin (10 µg), OF = Ofloxacin (5 µg), E = Erythromycin (10 µg), CT = Ceftriaxone (30 µg) and CXM = Septrin (30 µg).

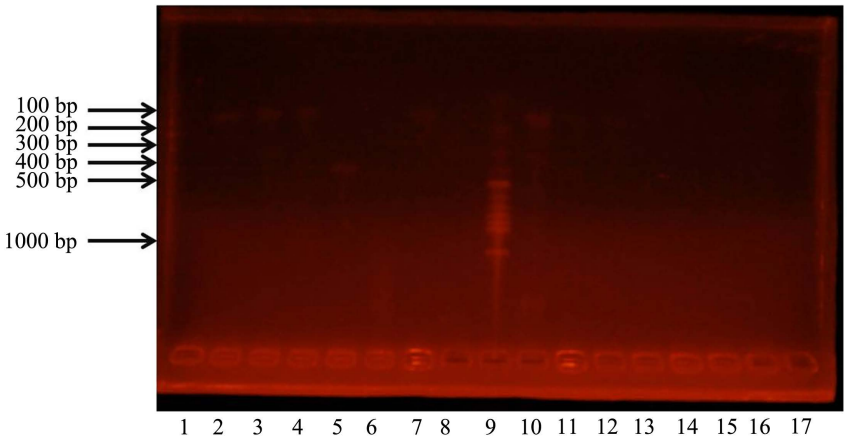

Figure 3. Multiplex PCR of clinically isolated MRSA strains using gene specific primer and genomic DNA preparation for MRSA typing. Lane 1: No MRSA gene, Lane 2: 162 bp of *mecA* gene, Lane 3: 303 bp for type III MRSA gene, Lane 4: 162 bp of *mecA* gene, Lane 5: 414 bp for type III MRSA gene, Lane 6: No MRSA gene, Lane 7: 162 bp of *mecA* gene, Lane 8: No MRSA gene, Lane 9: 100 bp DNA ladder, Lane 10: 303 bp for type III MRSA gene, Lane 11: 162 bp of *mecA* gene, Lane 12: 162 bp of *mecA* gene, Lane 13: 414 bp for type III MRSA gene. Lanes 14 - 17 showed absences of *mec* genes.

Table 3. MIC of drugs against isolates of MRSA.

Isolates	MIC (µg/ml)				
	Vancomycin	Clindamycin	Ciprofloxacin	Gentamycin	Ceftriaxone
1	250	250	31.25	13,330	4
2	250	250	62.5	675	4
3	125	125	31.25	675	2
4	125	250	62.5	42.12	0.5
5	125	62.5	62.5	675	2
6	250	31.25	15.63	675	2
7	250	31.25	62.5	42.12	0.5
8	250	187.5	31.25	13,330	4
9	125	375	62.5	675	2
10	125	375	31.25	675	2
11	62.5	23.44	62.5	42.12	0.5
12	125	375	31.25	675	2

4. Discussion

The distribution of MRSA isolates in the various clinical specimens suggests the strain as the most populous organisms in the clinical samples investigated. This is in agreement with the findings of some researchers, which indicated that MRSA may colonize a number of other body locations [6]. The biochemical test result confirmed the isolates as possible *S. aureus* and pathogenic [28]. Distinct

types of infections and patterns of antimicrobial susceptibility have been noted among other species of *Staphylococcus* [29]. On this basis, it is of clinical and epidemiological importance to identify these isolates, the specie and strain to avoid complicating the arduous challenge of antibiotic resistance via wrong reporting of other species as *S. aureus* especially, in developing and resource poor settings such as ours.

Result of inhibitory effects of these standard drugs (vancomycin and clindamycin) against isolates of MRSA is contrary to the findings of earlier reports [30] [31] which stated that these antibiotics have been used in the treatment of MRSA infections. This may be attributable to the constant genetic mutation of strains of MRSA thus making them resistant to many drugs over time. The appearance of antibiotic resistant bacteria has been regarded as an inevitable genetic response to the strong selective pressure imposed by antimicrobial chemotherapy, which plays an important role in the evolution of antibiotic resistant bacteria.

However, our findings support the reports of some works and the alarmingly increasing emergence of vancomycin and clindamycin resistance of *S. aureus* worldwide [30] [32]. Most *S. aureus* isolates, including MRSA, are still susceptible to vancomycin because it is not available for routine clinical use in Nigeria [33] [34]. The rate by which microorganisms grow resistance to antibiotics is always rapid, as such, antibiogram evaluation of *S. aureus* is very important and helps our understanding of emerging and re-emerging resistance trends and the development of appropriate therapeutic strategies in the management of MRSA infections [35].

The antibiotic sensitivity study on isolates of MRSA was carried out to ascertain the resistance trends of these organisms from different clinical isolates, to some antibiotics that are commonly used in the treatment of the patients. This study observed a relationship between methicillin resistance and resistance to other antibiotics. Others have similar result as well [32] [33]. To further ascertain the resistance of these isolates to the antibiotics, MIC was carried out. It was found from our result that the isolates were highly multi-resistant.

It can be deduced from the combination of results that clindamycin and vancomycin are not the best combination for the treatment of infections involving MRSA. It has been reported, that clindamycin frequently antagonizes the anti-staphylococcal activity of vancomycin [34]. Although, antagonism in drug combinations should be avoided in patient treatment, it has been opined that antibiotic antagonisms aid in serendipitous uncovering of treatment strategies that can delay the emergence of resistance [35]. Thus, the advantages of synergism and the different, uneventful results of antagonism helps understand how best to employ drug combinations in patient treatment. Despite the findings, drug antagonism still needs more studies to demonstrate usefulness as clinical options.

As there is an increase in the emergence and rapid dissemination of resistance to vancomycin and other antibiotics that has become a challenge to the treatment of human diseases, it was felt necessary to screen for the presence of resistance or resistance like DNA sequences that are present in various organisms

prior to carrying out molecular characterization. The *mecA* gene is harbored in a freely moving genetic element (sequences of genetic material) called staphylococcal cassette chromosome *mec* (SCC*mec*) [1] [36]. This is the reason why chromosomal DNA was extracted from MRSA cultures and used as a template for amplification. To verify the efficiency of the amplification and the absence of significant PCR inhibition, the qualities of the extracted DNA were checked. *S. aureus* isolates harboring SCC*mec* I, II and III genes were reported to come from healthcare-associated clones (HA-MRSA) and the allelic gene opined to be multiple resistance determinants, while type IV belongs to community-associated clones [1] [37]. Another study stated that in type I SCC*mec*, *mecA* gene was the only allelic resistance determinant gene, while in the SCC*mec* types II and III, multiple determinant allele is for resistance against antibiotics lacking β-lactam rings and are hospital-associated [38]. This is in concordance with our work and confirms that the isolates are basically H-MRSA. H-MRSA is typically resistant to many of the non-beta-lactam agents and also multi-drug resistant. Results obtained in this study showed that the broth microdilution method correlated excellently with the identification of the genetic determinants using multiplex PCR for *S. aureus* resistance to clindamycin and vancomycin. This shows that the majority of SCC*mec* cases are present in health institutions within the region.

5. Conclusions

Clindamycin and vancomycin-resistant MRSA infections are also within the Eastern region of Nigeria as found in other countries of the world. This superbug, therefore, may require drastic and urgent measures to curtail its spread and attendant healthcare challenges like outbreaks of infections and heightened healthcare delivery. In addition, strict adherence to antibiotic policy and continuous surveillance is highly advocated. Importantly focused research and development of new classes of anti-MRSA drugs will play a very important role in addressing drug resistance.

Strict adherence to standard hygienic practices such as regular hand washing, sterilization of hospital equipment as well as avoidance of close contact with patients is highly recommended.

Acknowledgements

The authors are grateful to the staff of the departments in all the hospitals and the laboratory where the research was conducted.

Authors' Contributions

MGUA, AAA and VCO, conceptualized and designed the study; MGUA, data collection; ANO and MGUO, data analysis; CEE, OIE and JE, writing, editing and proofreading. All the authors read and approved the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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